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PRIMER NOTE

EST-SSR markers from *Fragaria vesca* L. cv. Yellow Wonder

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Abstract

Fourteen microsatellite primer pairs were developed from a cDNA library of heat-treated seedlings of *Fragaria vesca* cv. yellow wonder. Transferability to 13 species of *Fragaria* ranged from 71% in diploid species *F. gracilis* Losinsk., *F. iinumae* Makino, *F. nilgerrensis* Schltdl. ex J. Gay and *F. nipponica* Makino, to 100% in octoploid domestic strawberry and its progenitors. Polymorphism was high in polyploid *Fragaria* species. However, polymorphism and heterozygosity of eight EST-SSRs (expressed sequence tag–simple sequence repeats) was low in 14 *F. vesca* genotypes.

Keywords: microsatellite, strawberry

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Fragaria vesca, the alpine or wild strawberry, is a self-compatible diploid perennial plant with widespread distribution throughout Eurasia and North America (Hancock 1999). Its small genome size (164 mbp) and possible contribution to the genome of the octoploid domestic strawberry, *Fragaria* × *ananassa* Duch., make it an ideal genome for comparative studies in *Fragaria*.

Genomic simple sequence repeats (SSRs) have been developed in *F. vesca* most recently by Monfort *et al*. (2005). We report the first set of EST-SSRs in *F. vesca*. In theory, EST-SSRs should show higher transferability as opposed to genomic SSRs, which would enable widespread use in mapping and comparing synteny across different ploidy levels in *Fragaria*. Our objective was to develop EST-SSR markers from a 'yellow wonder' EST library for use in cross-amplification in 13 species that encompass four ploidy levels ($2n$ = 14, 28, 35 and 56). Eight primer pairs were further used to identify 14 *F. vesca* accessions held at the US Department of Agriculture, Agricultural Research Service, National Clonal Germplasm Repository (NCGR).

SSR primer pairs were derived from a cDNA library of heat-treated seedlings of *Fragaria vesca* ssp. *vesca* f. *semperflorens* cv. Yellow Wonder. The library was constructed commercially in the pCMV.SPORT6.1 vector (Invitrogen). Approximately 1500 clones were sequenced (Virginia Bioinformatics Institute), and sequences were annotated at the ESTAP website (http://staff.vbi.vt.edu/estap/index.shtml).

The PBC Public website (http://hornbill.cspp.latrobe.edu.au/cgi-binpub/ssrprimer/indexssr.pl) or PRIMER 3 (Rozen & Skaletsky 2000) was used to design primers that amplified SSRs detected by PBC (Table 1). Nineteen primer pairs were designed to amplify repeats ≥ 14 bp in length. The design criteria included a polymerase chain reaction (PCR) product length of 100 and 350 bp. Additional parameters included a T_a of 57–64 °C (optimum 60 °C), and a primer length of 20–25 bp (optimum 22 bp). Primer pairs were denoted UAFv (USDA/ARS *F. vesca*) followed by a four-character numeric EST code (i.e. UAFv 0000) (Table 1).

Each primer pair was used to amplify DNA from 14 accessions of *F. vesca* (list provided upon request) and 36 other *Fragaria* species including one representative of *F. daltoniana* J. Gay (PI 641195), octoploid *F. iturupensis* Staudt (PI 641091) and *F. nubicola* (Hook. f.) Lindl. ex Lacaita (PI 551851); two accessions each of *F. gracilis* (PI 616583, PI 551576), hexaploid *F. moschata* Weston (PI 551528, PI 551549), *F. nilgerrensis* (PI 602577, PI 616672) and *F. viridis* Weston (PI 616611, PI 551742); three genotypes each of *F. nipponica* (PI 637974, PI 637975, PI 637976) and *F. pentaphylla* Losinsk. (PI 637926, PI 641194, PI 637926) and tetraploid *F. orientalis* Losinsk. (PI 602942, PI 551864, PI 637933); and four representatives of *F. iinumae* (PI 637963, PI 637964, PI 551751, PI 616505) and of the octoploid species *F. chiloensis* (L.)

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Table 1 Fourteen *F. vesca* EST-SSR forward (F) and reverse (R) primer pairs, motifs, optimum annealing temperatures (T_a), expected size of the PCR product in bp, and genes that were identified by BLAST hits and the *E*-value. Eight EST-SSR primer pairs that appeared polymorphic in *F. vesca* were further used to identify 14 *F. vesca* genotypes. Allele number and size range for these eight SSRs are listed. The number of alleles at locus UAFv9768, which also appeared polymorphic in *F. vesca*, was not determined (ND) by capillary electrophoresis

SSR locus	Repeat motif	Primer sequences	T_a (°C)	Exp. size (bp)	Size range	Allele no.	Gene (Accession no.)	*E*-value
UAFv7344	$(TTCT)_4$	F: TCCTTTGTTTATTTGTATTGTTT R: ATGATTGAAGTGGTGAAGATG	50	202		1	*Aralia cordata* cadac1 mRNA for cinnamyl alcohol dehydrogenase (gi|451196)	5.00E-49
UAFv7500	$(CTC)_6$	F: GTGAGAACACTAACACCACCA R: GGATTTGAGGAGGGAGAA	58	326		1	*Arabidopsis thaliana* zinc finger (C3HC4-type RING finger) family protein (gi|30691621)	1.00E-15
UAFv7648	$(CT)_{12}$	F: AACCAGAGCCAGAGCCAG R: CGACAGTGATGTAGAGGAAGA	63	238	235–239	3	*Brassica oleracea* aquaporin (PIP3) mRNA (gi|11119334)	1.00E-57
UAFv8150*	$(CTC)_6$	F: CCACCTCTCTCTCCATTTCC R: AGCGGTGTGAAGACTTGAGG	58	219		1	*A. thaliana* metallo-beta-lactamase family protein mRNA (gi|42568732)	3.00E-71
UAFv8204	$(CT)_{11}$	F: CTCTGCCTTTCTCTACCC R: CCCAAGTCTATGAGTGGAAC	48	245	239–272	5	*Malus* × *domestica* ap20ox gene for gibberellin 20-oxidase (gi|12231167)	8.00E-46
UAFv8216*	$(GGC)_6$	F: GGTAATGCAGCACCAAATGA R: GGAAGCGAAGCAGTTATGGA	57	235	213–231	3	*Nicotiana tabacum* mRNA for nucleic acid binding protein (nbp1 gene) (gi|15594034)	2.00E-09
UAFv8316*	$(TC)_8$	F: CGGTTAAACCAGATTACAACTCTC R: GATCGAGCCCTACCAATTCA	62	235	229–233	3	*A. thaliana* endonuclease/exonuclease/phospha-tase family protein mRNA (gi|18410988)	8.00E-12
UAFv8936	$(TA)_7$	F: GTGACTTTGACGCTGACC R: TGAGAGTGGTTCTGTTCCTC	62	310	310–312	2	*A. thaliana* expressed protein (gi:18378999)	3.00E-22
UAFv9092	$(AGA)_6$	F: ACCACAATCCTCCGCCATT R: AGTCGTGCTTGATGTTGAG	62	314	310–312	2	*A. thaliana* similar to dihydroflavonol reductase mRNA (gi|14596184)	5.00E-81
UAFv9404*	$(ATG)_7$, $(CTC)_7$	F: AGTCGTGCATCATGGATCAG R: CATTAGTTGGCCACACACCA	66	298		1	*A. thaliana* PHD finger protein — like (gi|15239453)	9.00E-17
UAFv9574	$(AG)_{11}$	F: AGAGAACAGAGAGCCAGAAAC R: GAATGGGAAGAAGGAGGA	52	262	260–274	3	*A. thaliana* phosphatidylglycerolphosphate synthase (PGS1) mRNA (gi|42569763)	2.00E-15
UAFv9588	$(GTG)_7$	F: TTTCTCTCTCCCTTTCACTCT R: GACCACCATCTCTCCTGTAA	50	335		1	*A. thaliana* DNA-binding protein (gi|21537255)	6.00E-72
UAFv9690	$(AG)_{10}$	F: CAGAGAGCGAGAGAGTCAAA R: GAGATTGGTTGGGACAGAC	58	189	184–188	3	*A. thaliana* putative OsNAC6 protein mRNA (gi|15292730)	2.00E-43
UAFv9768	$(TTTA)_4$	F: CTCAACTACGCCACGCCC R: AAGCAATCCATACAGAACAGA	62	280		ND	No significant similarity	

*indicates that PRIMER 3 was used for primer design as opposed to PBC Public used for the remaining loci.

Table 2 Cross amplification (A) and polymorphism (P) of EST-SSRs in 14 *Fragaria* species

Locus	Amplification/polymorphism* in *Fragaria* species														
	Diploid									Tetraploid	Hexaploid	Octoploid			
Ploidy	*nil*	*gra*	*vir*	*pent*	*nip*	*nub*	*dalt*	*iinu*	*ves*	*ori*	*mos*	*chil*	*itr*	*virg*	*ana*
UAFv7344	+/–	+/–	+/–	+/–	+/–	+	+	+/–	+/–	+/–	+/–	+/–	+	+/–	+/–
UAFv7500	+/–	+/–	+/+	+/–	+/+	+	+	–/–	+/–	+/+	+/–	+/+	+	+/+	+/+
UAFv7648	+/–	+/–	+/–	+/+	+/+	+	+	+/–	+/+	+/+	+ +	+/+	–	+/+	+/+
UAFv8150	+/–	+/–	+/+	+/–	–/–	+	+	+/–	+/–	+/+	+/+	+/+	+	+/+	+/+
UAFv8204	+/–	+/+	+/+	+/+	+/+	+	+	+/+	+/+	+/+	+/+	+/+	+	+/+	+/+
UAFv8216	+/–	+/–	+/+	+/+	+/+	+	+	+/–	+/+	+/+	+/–	+/–	+	+/+	+/+
UAFv8316	+/–	+/–	+/+	+/–	+/+	–	+	+/–	+/+	+/–	+/–	+/+	+	+/+	+/+
UAFv8936	+/+	+/+	+/+	+/+	+/+	+	–	+/+	+/+	+/+	+/+	+/+	+	+/+	+/+
UAFv9092	+/+	+/+	+/+	+/+	+/+	+	+	+/–	+/–	+/+	+/–	+/+	+	+/+	+/+
UAFv9404	–/–	–/–	+/–	–/–	+/–	+	+	+/–	+/–	+/+	+/+	+/+	+	+/+	+/+
UAFv9574	–/–	+/+	+/+	+/+	+/+	+	+	–/–	+/+	+/+	+/+	+/+	+	+/+	+/+
UAFv9588	–/–	–/–	+/–	–/–	–/–	+	–	–/–	+/–	+/–	+/–	+/–	–	+/–	+/–
UAFv9690	+/–	–/–	+/+	+/+	–/–	+	+	–/–	+/+	+/+	+/–	+/–	+	+/+	+/+
UAFv9768	–/–	–/–	+/+	–/–	–/–	–	–	+/–	+/+	+/+	+/+	+/+	+	+/+	+/+

*Amplification/polymorphism: Amplification, + indicates a product was amplified, – indicates lack of amplification; Polymorphism, +indicates polymorphism, – indicates lack of polymorphism.
Abbreviations include *nil* for *F. nilgerrensis* (PI 602577, PI 616672), *gra* for *F. gracilis* (PI 616583, PI 551576), *vir* for *F. viridis* (PI 616611, PI 551742), *pent* for *F. pentaphylla* (PI 637926, PI 641194, PI 637926), *nip* for *F. nipponica* (PI 637974, PI 637975, PI 637976), *nub* for *F. nubicola* (PI 551851), *dalt* for *F. daltoniana* J. Gay (PI 641195), *iinu* for *F. iinumae* (PI 637963, PI 637964, PI 551751, PI 616505), *ves* for *F. vesca* (list of accessions provided upon request), *ori* for *F. orientalis* (PI 602942, PI 551864, PI 637933), *mos* for *F. moschata* (PI 551528, PI 551549), *chil* for *F. chiloensis* (PI 612318, PI 612489, PI 612487, PI 616934), *itr* for *F. iturupensis* (PI 641091), *virg* for *F. virginiana* (PI 612491, PI 612486, PI 551527, PI 612492), and *ana* for *F.* × *ananassa* (PI 551400, PI 551842, PI 551620, PI 551594).

Mill. (PI 612318, PI 612489, PI 612487, PI 616934), *F. virginiana* Mill. (PI 612491, PI 612486, PI 551527, PI 612492), and *F.* × *ananassa* (PI 551400, PI 551842, PI 551620, PI 551594).

PCRs were performed in 10 μL volume containing 1× reaction buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 μM of each primer, 0.25 U of Biolase *Taq* DNA polymerase (Bioline), and 2.5 ng genomic DNA. The PCR protocol consisted of one cycle of initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 93 °C for 40 s, annealing at optimum T_a for 40 s, and extension at 72 °C for 40 s. A final extension cycle at 72 °C for 30 min followed. DNA was amplified in an Eppendorf Gradient thermocycler (Brinkmann Instruments) or an MJ Research Tetrad thermocycler. PCR products were separated in 3% agarose gels and scored for amplification and polymorphism after visualization by ethidium bromide staining (Table 2). The number of apparent alleles of eight EST-SSRs was determined in 14 *F. vesca* accessions using fluorescently labelled forward primers (Table 1) after separating the PCR products by capillary electrophoresis using the Beckman CEQ 8000 genetic analyser (Beckman Coulter). Tests for linkage disequilibrium (LD) and deviations from Hardy–Weinberg equilibrium (HWE) were performed using GENEPOP on the web (Raymond & Rousset 1995; http://wbiomed.curtin.edu.au/genepop/).

Fourteen of the 19 primer pairs (73.6%) amplified the expected size fragment in *F. vesca*. Cross amplification in diploid species ranged from a low of 71% in *F. gracilis*, *F. iinumae*, *F. nilgerrensis* and *F. nipponica* to 100% in *F. viridis* and *F. vesca*. Amplification in the remaining diploid species was intermediate: 78% in *F. daltoniana* and *F. pentaphylla*. The 14 SSR primer pairs resulted in 100% amplification in tetraploid, hexaploid and octoploid *Fragaria* species except for *F. iturupensis*, where only 12 primer pairs (85%) generated a PCR product. In diploid *Fragaria*, polymorphism ranged from 14% in *F. iinumae* and *F. nilgerrensis* to 57% in *F. nipponica* and *F. vesca*. In polyploid species however, polymorphism ranged from 50% in *F. moschata* to 85% in *F. virginiana* and *F.* × *ananassa*.

The number of apparent alleles (PCR products) of eight EST-SSRs in 14 genotypes of *F. vesca* ranged from two to five per primer pair, averaging three (Table 1). These eight EST-SSRs could not differentiate between three pairs of genotypes ('Yellow Solemacher' and 'Golden Alpine'; 'Pineapple Crush' and 'Alexandria'; 'Baron Solemacher' and 'Rodluvan'). Observed heterozygosity (H_O) ranged from 0 to 0.07, whereas expected heterozygosity (H_E) ranged

from 0.13 to 0.76. A significant deviation from HWE ($P < 0.05$) was observed at each locus. Significant LD ($P < 0.05$) was found between six pairs of loci: USFv7648 and USFv8216; USFv7648 and USFv9690; USFv8216 and USFv9690; USFv7648 and USFv8204; USFv8216 and USFv8204; USFv9690 and USFv8204.

Low heterozygosity, lower H_O, as opposed to H_E, and significant deviation from HWE observed in this study are consistent with high amount of inbreeding reported in *F. vesca* (James *et al*. 2003).

High cross-species transference of these EST-SSRs in the *Fragaria* genus will increase their usefulness for linkage mapping and comparative studies.

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